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TITLE: DEVELOPMENT OF SEROLOGIC ASSAYS FOR THE DIAGNOSIS OF NEW WORLD LEISHMANIASIS

PRINCIPAL INVESTIGATOR: Ronald L. Anthony, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
School of Medicine
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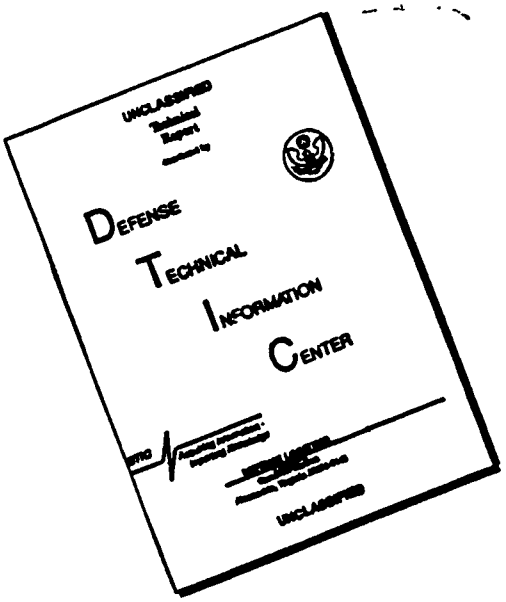

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Leishmania; monoclonal antibodies; amastigotes; serodiagnosis; flow cytometry; immunopathology. (EDC)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Genus-specific monoclonal antibodies have been generated to 14 different isolates of New World Leishmania. These antibodies have been used to identify closely related subspecies: to recover genus-specific antigens for the development of serodiagnostic assays; and to identify parasites in infected tissues; to quantitate antigen expression on the surface membrane by flow cytometry. Keywords:

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SUMMARY:

Major activities conducted during the second year of the contract DAMD 17 - 83 C-3031 included:

1. Increasing our inventories of monoclonal antibodies to the New World Leishmania.

2. Use of the monoclonal antibodies for the identification and recovery of species-, strain- and stage-specific antigens.

3. Use of the specific antigens for development of species-specific serodiagnostic assays.

4. Use of the monoclonal antibodies to detect parasites in infected tissues.

5. Evaluation of techniques of flow cytometry to quantitate surface antigen expression by the parasites and to monitor the effect of the monoclonal antibodies on host cell-parasite interactions.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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TABLE OF CONTENTS

PROGRESS REPORT:

1. ESTABLISHMENT OF INVENTORIES:

A. PARASITES:

In vitro cultures of 14 different isolates of New World trypanosomatids were established in our laboratory. Adequate stocks have been stored in liquid nitrogen.

WRAIR 2028.....	Leishmania	mexicana	mexicana
WRAIR 301.....	Leishamnia	mexicana	amazonensis
GML 111.....	Leishmania	mexicana	amazonensis
GML 003.....	Leishmania	mexicana	aristides
WRAIR 140.....	Leishmania	peruviana	
WRAIR 470.....	Leishmania	braziliensis	panamensis
WRAIR 390.....	Leishmania	braziliensis	panamensis
GML 001.....	Leishmania	braziliensis	panamensis
GML 018.....	Leishmania	braziliensis	braziliensis
WRAIR 359.....	Leishmania	braziliensis	braziliensis
WRAIR 508.....	Leishmania	braziliensis	braziliensis
WRAIR 484.....	Leishmania	donovani	chagasi
Tulahuen	Trypanosoma	cruzi	
GML 465.....	Endotrypanum	schaudinni	

B. MONOCLONAL ANTIBODIES:

In addition to the monoclonal antibodies described in Annual Report No. 1B , covering activities from December 1, 1982 to November 30, 1984, the following monoclonal antibodies have now been added to our inventories.

Fusion	Immunogen	No. of monoclonal antibodies
84C	WRAIR - 303 (insect forms)	7
84E	WRAIR - 50R (promastigotes)	1
84F	WRAIR - 303 (insect forms)	26
84G	GML - 1 (promastigotes)	5
84J	GML - 18 (promastigotes)	8

SPECIFICITY OF MONOCLONAL ANTIBODIES AND RECOVERY OF ANTIGENS:

Fusion 84C: Results of indirect immunofluorescent antibody assays.

MONOCLONAL ANTIBODY	ISOLATE				
	470	303	222b	508	T. cruzi
84C-1F2-surface	4+	2+	4+	2+	3+
84C-2G9 surface	+/-	2+	neg	2+	1+
84C-3D6-surface	+/-	2+	neg	3+	2+
84C-4F4-surface	4+	1+	4+	1+	neg
84C-5B2-surface	4+	4+	4+	4+	neg
84C-8B3-surface	3+	3+	4+	3+	neg
84C-8C7--surface	3+	neg	3+	neg	neg

84F--Immunogen= Sandfly form of WR303 (L.m. amazonensis)

ELISA data:Optical Density at 405 nanometers.

Values represent means of a minimum of 3 assays performed on alternate days with different lots of antigen.

Isolates*	Monoclonals		
	4G5	9B6	9D11
Site of reactivity	Nucleus	Pocket	Surface
470	0.498	0.054	0.528
TC	0.460	0.089	0.457
390	0.452	0.047	0.417
16	0.330	0.062	0.336
140	0.402	0.029	0.432
222	0.515	0.052	0.511
359	0.479	0.072	0.458
3	0.406	0.045	0.380
484	0.376	0.026	0.398
465	0.347	0.039	0.396
111	0.459	0.046	0.408
508	0.540	0.081	0.518
303*	0.562	0.048	0.547
1	0.515	0.070	0.488

(A) All monoclonals from fusion 84F lacked specificity at the genus-level:

(B) Additional investigations are not planned at this time.

(C. 84F-465 was interesting in that its pattern of immunofluorescence was not seen previously.

(D) *=homologous reaction

84G--Immunogen= stationary promastigotes of isolate GML-1 (L. b panamensis)

Isolates	Monoclonals			
	G6B6	G8B10	G9E2	G9G3
Site of Reactivity	Surface + Flagellum	Surface	Cytoplasmic Granules	Pocket + Flagellum
470	0.465	0.037	0.195	0.109
FC	0.374	0.003	0.135	0.063
390	0.534	0.015	0.137	0.085
18	0.370	0.021	0.127	0.062
140	0.264	0.028	0.192	0.063
222	0.465	0.016	0.208	0.094
359	0.414	0.050	0.181	0.111
3	0.365	0.022	0.145	0.051
484	0.396	0.004	0.168	0.080
465	0.332	0.005	0.127	0.056
111	0.453	0.029	0.176	0.077
508	0.569	0.019	0.223	0.136
303	0.500	0.041	0.252	0.098
1*	0.426	0.140	0.247	0.105

(A) Antibody G8B10 appears to be specific for isolate GML-1.

(B) Minimal reactivity was also seen with two other L. braziliensis sp. (359 and 470).

(C) *= homologous reaction

b braziliensis)

Isolates*	Monoclonal Antibodies							
	J3D2	J3G8	J4D10	J6B9	J6B11	J8E6	J8G10	J9C5
Site of	Flag.	Surf.	Surf.	Surf.	Surf.	Surf.	Surf.	Surf.
Rectivity								
470	0.149	0.035	0.086	0.064	0.104	0.063	0.066	0.042
TC	0.153	0.000	0.042	0.028	0.087	0.043	0.022	0.005
390	0.117	0.008	0.059	0.068	0.088	0.086	0.015	0.012
18*	0.128	0.043	0.042	0.049	0.090	0.053	0.080	0.033
140	0.133	0.037	0.020	0.065	0.052	0.025	0.020	0.013
222	0.138	0.019	0.091	0.040	0.059	0.027	0.045	0.031
359	0.164	0.027	0.045	0.094	0.106	0.093	0.059	0.019
3	0.112	0.005	0.050	0.027	0.072	0.006	0.004	0.004
484	0.124	0.002	0.043	0.045	0.071	0.006	0.006	0.017
465	0.071	0.010	0.050	0.029	0.056	0.015	0.015	0.000
111	0.142	0.019	0.091	0.073	0.089	0.054	0.014	0.013
508	0.161	0.028	0.124	0.075	0.127	0.070	0.070	0.015
303	0.172	0.032	0.115	0.067	0.071	0.025	0.080	0.009
1	0.188	0.018	0.120	0.066	0.112	0.032	0.066	0.012

(A) Reactivity of all antibodies was minimum in ELISA.

(B) Antibody J8G10 exhibits some specificity for the braziliensis complex.

(C) Antibody J9C5 may proove to be genus-specific.

(D) Antibodies J4D10 and J6B11 recognize non-specific surface antigens.

(E) Continued evaluations of this panel are in progress.

We have continued to direct our major effort towards separating the 14 isolates according to genus, species, and subspecies on the basis of their reactivity with a panel of monoclonal antibodies. Reactivity was assessed using a solid-phase ELISA wherein the antigens were air-dried promastigotes attached to poly-l-lysine coated microtiter plates. The following table is a summary of results. + = optical density >0.075 at 405 nm. * = homologous reaction.

	ISOLATE													
	222	303	111	3	140	470	390	1	18	359	508	484	TC	ES
MAB														
H2D6	+*	+	+	+	+	+	-	-	-	+	+	+	-	-
L2D3	+	+	+		+	+*	+	+	-	-	+	+	-	-
L5J9	+	+	-	-	+	+*	-	-	-	-	-	+	-	-
T5A9	+*	+	-	-	+	+	-	-	-	-	-	+	-	-
T5C6	+*		-	-	+	+	-	-	-	-	-	+	-	-
T7D3	+*	+	-	-	+	+	-	-	-	-	-	+	-	-
T10C4	+*	+	-	-	+	+	-	-	-	-	-	+	-	-
U3F11	+*		-	-	-	-	-	-	-	-	-	-	-	-
U3F2	-*	-	-	-	-	-	-	-	-	-	-	-	-	-
U7D5	+*	+	-	-	+	+	-	-	-	-	-	+	-	-
U9B2	+*	-		-	+	+	-	-	-	-	-	+	-	-
C1F2	+	+*	+	+	+	+	+	+	+	+	+	+	+	+
C4F4	+	+*	-	-	+	+	-	-	-	-	-	+	-	-
C5B2	+	+*	+	+	+	+	+	-	-	+	+	+	-	-
C8B3	+	-*	-	-	+	+	-	-	-	-	-	-	-	-
C8C1	-	-*	-	-	-	+	-	-	-	-	-	+	-	-
E1CC6	+	+	+	+	+	-	+	+	+	+	+*	+	+	+

Comments:

1. A good number of the negative reactions might be considered weakly positive (OD values in the 0.050 - 0.070 range). However, lowering the cut-off level to 0.050 does not improve specificities. It should be noted that the negative values for Tc and Es were consistently far below the 0.050 reading.

2. We do feel confident in our ability to discriminate at the genus level

3. The leishmania can be separated into two major groups at this time: Additional information on speciation (e.g. isoenzymes) is needed for all isolates.

Group #1	Group #2
222B	111
303	3
140	390
470	1
484	18
	359
	508

4. The assay is extremely reproducible. Although the data in the above table represents an average of two assays performed on different days, the reactivity of some monoclonals has been assessed as many as thirty times against 6 different isolates: Specificity does not vary. This data has been accepted as a poster for the December ASTMH in Baltimore.

5. The ELISA data is supported by IFA data. Specificities hold true for both assays.

6. We obviously need additional monoclonal antibodies against the braziliensis complex. Unfortunately, Fusion 84E (against 508) yielded only one stable antibody producer (E10C6) which lacked specificity at the genus level. Fusion 84G, against GML# 1, resulted in 314 hybridomas of which only 6 were antibody producers, as determined by IFA. These hybridomas are being screened by ELISA and are in the process of expansion. Specificity assays will be completed in October.

7. Antibody U7D5 (purified from ascitic fluid by affinity chromatography) has been used as an immunosorbent to recover the U7D5 antigen from deoxycholate extracts of 222B promastigotes. A pool of antigen has been made and preliminary experiments indicate that sera from human cases of leishmaniasis (Panamanians) contain antibody to the antigen. Immunochemical analyses of the antigen and assays for specificity are in progress.

8. The additional fusion (84F) was performed using splenocytes from a mouse immunized with insect forms of 303. Of 150 hybridomas, 26 were reactive with the 303 insect forms by IFA. Eight of the clones are being expanded and specificity assays will be compeleted within the next two weeks.

9. Mice have been immunized with GML 18; a fusion is planned during the week of September 17

10. We suspect that the conditions of culture may influence the surface antigen
composition of the particular isolate. Experiments to confirm or refute this suspicion are in progress.

11. Antibody L2D3 has been purified from ascitic fluid and affinity columns should be ready within the next few weeks.

CHARACTERIZATION AND PURIFICATION OF THE REACTIVE ANTIGENS .

Recovery of specific antigens recognized by monoclonal antibodies continues. These efforts entail production of ascitic fluids; purification of the fluids of Affi-gel Blue columns; construction of affinity columns (Affi-gel 10) using the purified monoclonal as the immunosorbent; solubilization of the antigen (promastigotes); elution of the solubulized antigen through the affininty column; characterization of the eluted fractions by Western Blots followed by radio-immunesprecipitation with the monoclonal antibody. The following studies are in progress.

Monoclonal Antibody	Specificity		Site of Reactivity	Antigen (kd)	Recovered
(ASCITES)					
U7D5	+	-	SURFACE	62,000	YES
	470	TC		65,000	
	390	465		DOUBLET	
	140	508			
	222*	1			
	359	18			
	484				
	111				
	303				
	3				

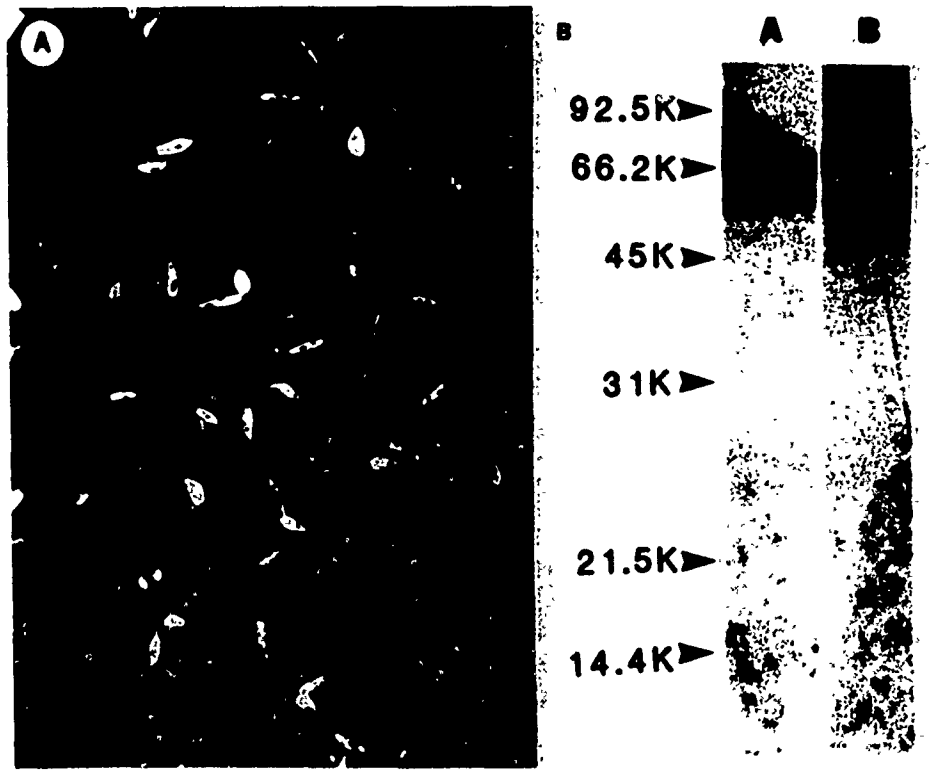
L2D3	+	-	SURFACE	14,000	YES
	470*	TC		15,000	
	390	18		60,000	
	140	465		TRIPLET	
	222	500			
	359	1			
	3				
	484				
	111				
	303				

*=homologous reaction

OTHER MONOCLONALS (ASCITES) UNDER INVESTIGATION INCLUDE:

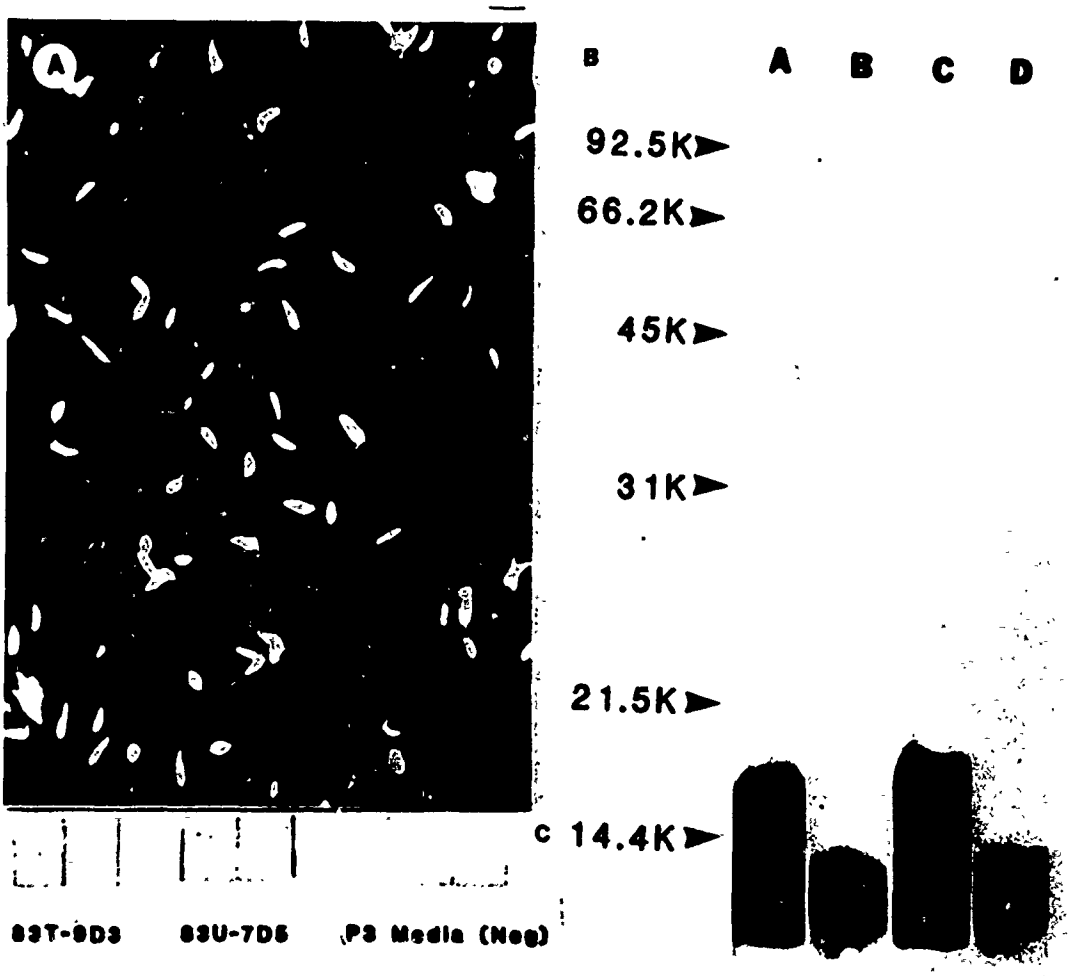
T9D3= REACTIVE WITH 15,000 kd antigen on surface of 222.

L5G9=	"	"	?	"	"	"	"	470
R5D2=	"	"	?	"	"	"	"	"
U3F2=	"	"	?	"	"	"	"	"
L9D6=	"	"	?	"	"	"	"	"
G8B10=	"	"	?	"	"	"	"	1
G9B3=	"	"	?	"	"	"	"	"
G6B6=	"	"	?	"	"	"	"	"
C4F4=	"	"	?	"	"	"	"	303
C8C7=	"	"	?	"	"	"	"	"
H2D6	"	"	67,000 kd	"	"	"	"	222
C5D2	"	"	"	"	"	"	"	303



A. Immunofluorescent micrograph demonstrating reactivity of monoclonal antibody 3H-2D6 with air dried promastigotes of isloate WRAIR 222b.

B. Immunoelectroblot verifying the specific reactivity of monoclonal antibody 3H-2D6 with a 67 kd protein of a crude extract of WRAIR 222b promastigotes.



A. Immunofluorescent micrograph demonstrating reactivity of monoclonal antibody 83T-9D3 with air dired promastigotes of isloate WRAIR 222b.

B. Immunoelectoblot verifying the specific reactivity of monoclonal anti dy 83T 9D3 with a 15 kd protein of a crude extract of WRAIR 222b prom stigotes.

3. DEVELOPMENT OF SERODIAGNOSTIC ASSAYS.

The surface antigen of WRAIR-470 isolate recognized by monoclonal antibody 83L-5G9 was recovered from extracts of stationary promastigotes by affinity chromatography (Figure 1). The genus specificity of that monoclonal antibody had been confirmed by enzyme linked immunosorbent assays (Figure 2).

Figure 3 represents the ELISA data when the reactivity of human sera, representative of leishmaniasis, trypanosomiasis and toxoplasmosis, was measured against the purified 83L-5G9 antigen.

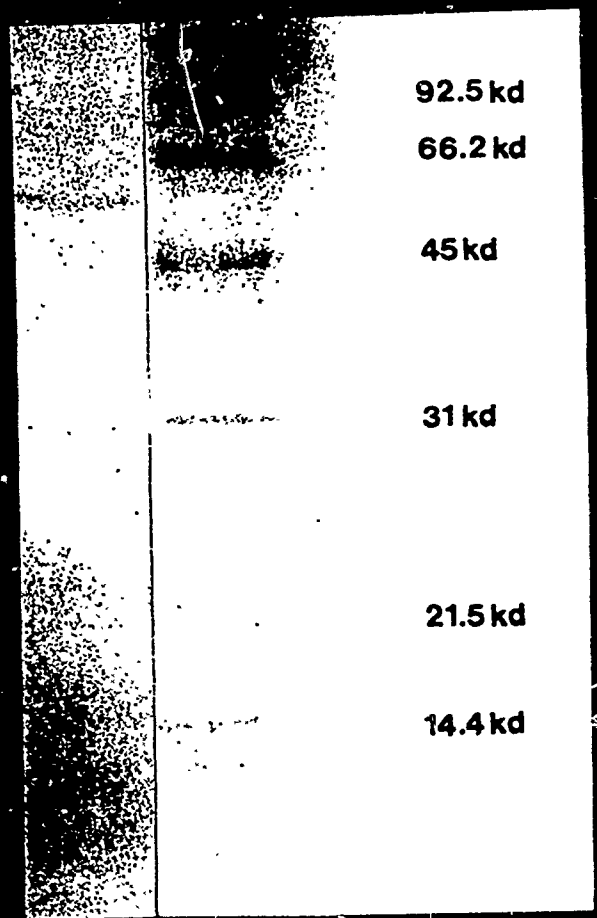


FIGURE 1 : SDS-PAGE OF 831-569 PURIFIED ANTIGEN REVEALING TWO BANDS OF 58 KD AND 31 KD.

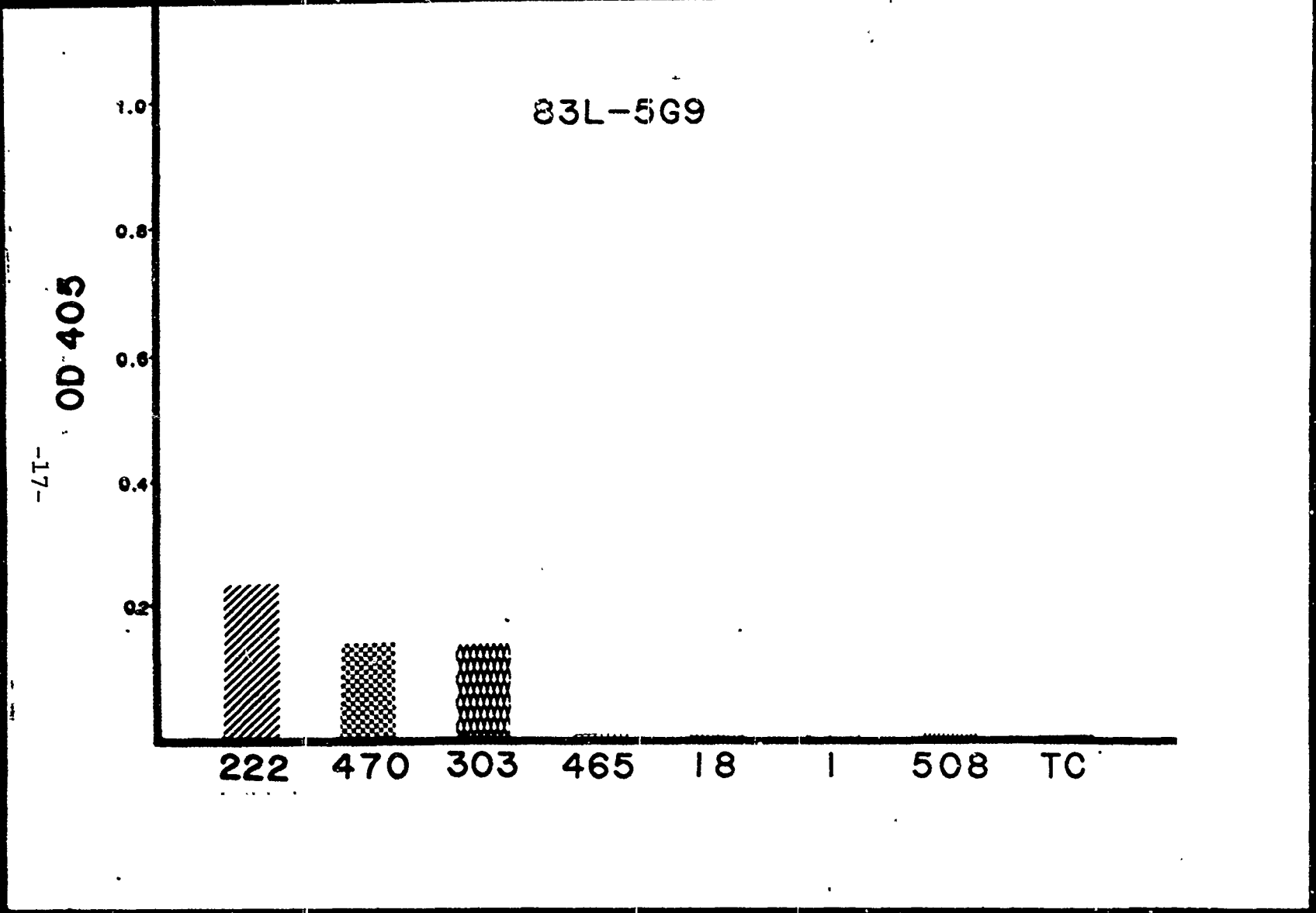


FIGURE 2: ENZYME LINKED IMMUNOSORBENT ASSAY RESULTS FOR 83L-569 ANTIBODY AGAINST VARIOUS PARASITE SPECIES.

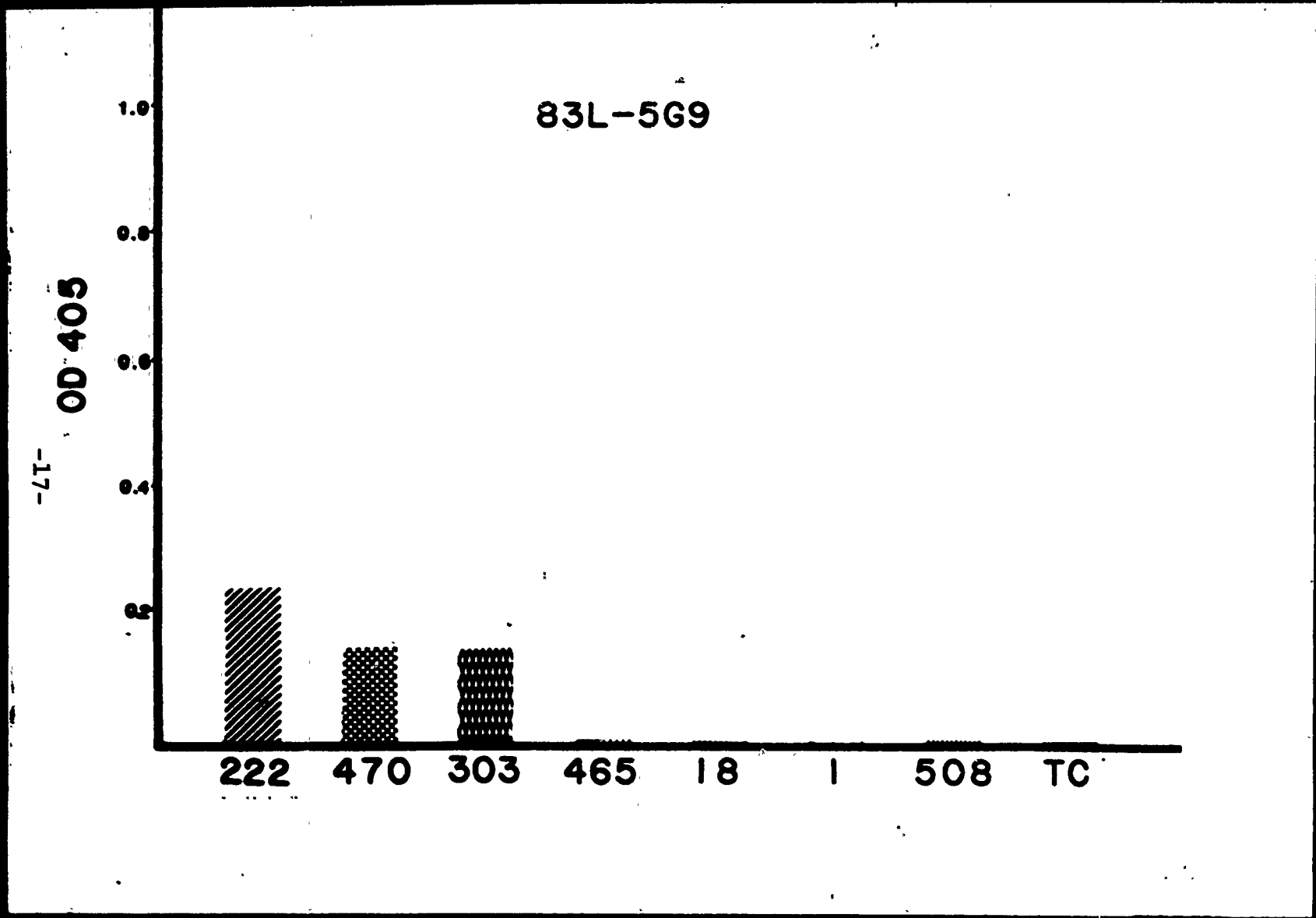


FIGURE 2: ENZYME LINKED IMMUNOSORBENT ASSAY RESULTS FOR 83L-569 ANTIBODY AGAINST VARIOUS PARASITE SPECIES.

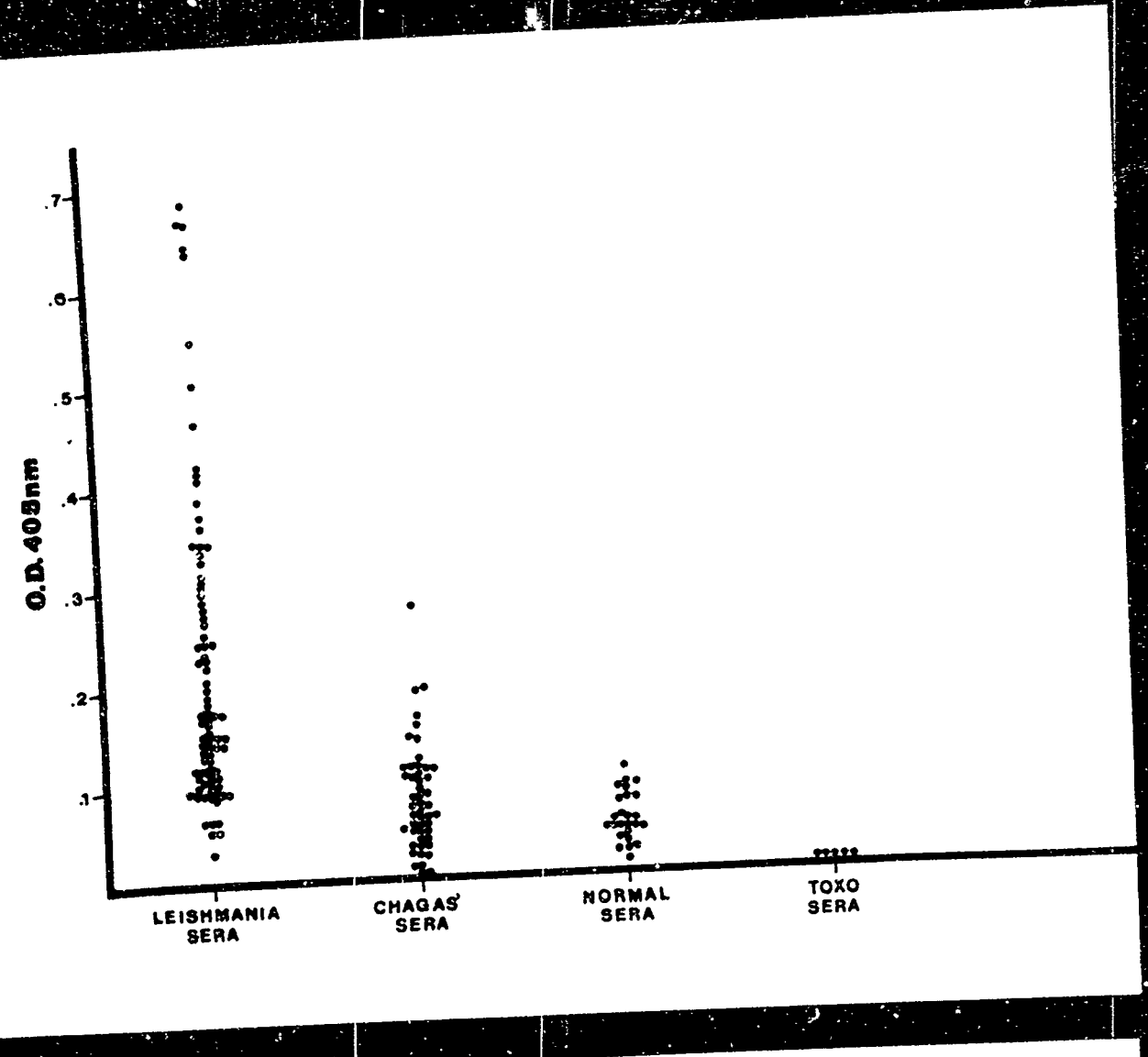


FIGURE 3. ENZYME LINKED IMMUNOSORBENT ASSAY RESULTS OF HUMAN SERA WHEN PURIFIED 83L-569 ANTIGEN IS USED TO COAT

4. IDENTIFICATION OF PARASITES IN INFECTED TISSUES:

Approximately 10,000 promastigotes of isolate GML 111, Leishmania mexicana amazonensis , were introduced into the footpads of 8-week old Balb/c mice. After one month, when all mice exhibited visible lesions, the nodule was excised and cut into 2mm cubes. Several cubes were cultured for retrieval of parasites and the remainder were embedded in OTC and snap frozen in liquid nitrogen. The frozen specimens were sectioned at 5 microns and after a brief fixation in 70% methanol, were used as substrates for indirect immunofluorescent antibody assays. The capability of the monoclonal antibodies to detect intracellular parasites in these infected tissues is presented in the following [illegible]

[illegible] monoclonal antibodies with amastigotes, representative of 8 isolates, is presented in the following table. These amastigotes were produced by the *in vitro* infection of mouse peritoneal macrophages.

TABLE I

REACTIVITY OF MONOCLONAL ANTIBODIES WITH AMASTIGOTES OF NEW WORLD LEISHMANIA SPECIES

MONOCLONAL ANTIBODY	*L. m. mexicana* (WR 222)	*L. m. amazonensis* (WR 303)	*L. m. amazonensis* (GML 111)	*L.* species (WR 359)	*L. b. guyanensis* (WR 390)	*L. b. panamensis* (GML 1)	*L. b. braziliensis* (WR 508)	*L. b. braziliensis* (GML 18)
83H-2D6	4+	4+	4+	4+	4+	4+	4+	-
83L-2D3	4+	4+	4+	4+	4+	-	4+	-
83L-5G9	4+	4+	4+	-	4+	-	-	-
83T-3E7	4+	4+	-	-	-	-	-	-
83T-3E9	4+	4+	4+	-	4+	-	-	-
83T-4D7*	-	-	-	-	-	-	-	-
83T-5C6*	-	-	-	-	-	-	-	-
83T-9D3	4+	4+	4+	-	-	-	-	-
83T-10E4	4+	4+	4+	-	4+	-	-	-
83U-2F11	4+	4+	4+	-	4+	-	-	-
83U-5F2	2+	2+	2+	-	-	-	-	-
83U-7D5	4+	4+	4+	-	-	-	-	-
83U-9B3	4+	4+	4+	-	-	-	-	-
84C-4F4	4+	4+	4+	-	4+	-	-	-
84C-5B2	4+	4+	4+	4+	4+	4+	4+	-
84C-8B3	4+	4+	-	-	-	-	-	-
84C-8C7	4+	4+	4+	-	4+	-	-	-
84G-8B10	-	-	-	-	-	4+	-	-

* Specific for *L. mexicana* promastigote membrane.

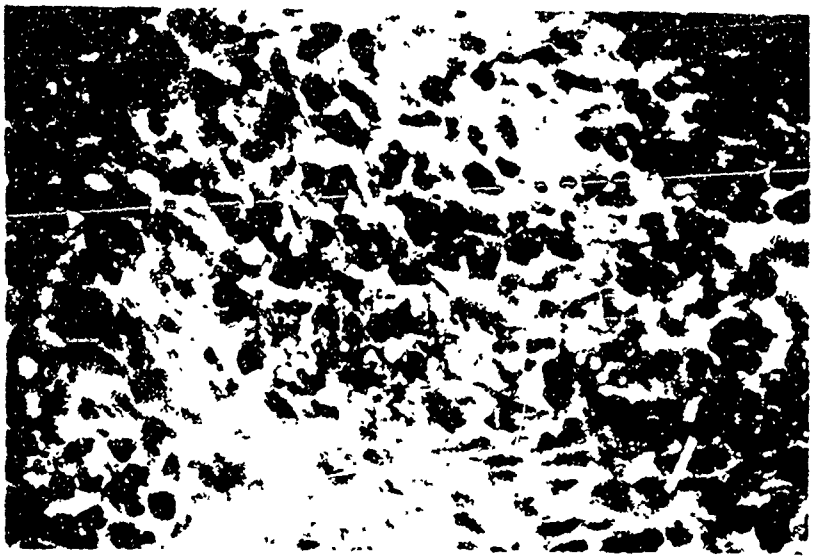
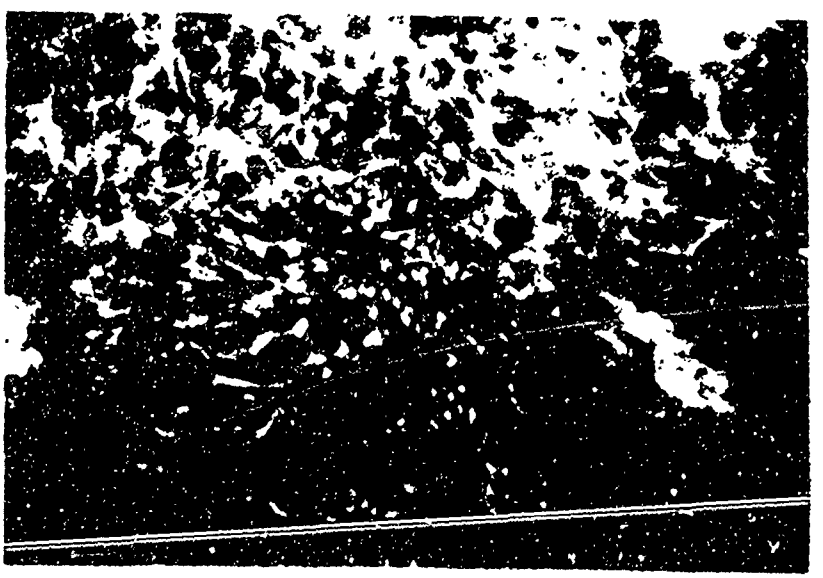


Fig. 1 Immunofluorescent identification of amastigotes in the footpad nodule of a Balb/c mouse 1 month after inoculation with L. m. amazonensis promastigotes. Frozen sections stained with L. mexicana-specific monoclonal antibody 83U-7D5 as described in Materials and Methods. Most amastigotes are localized to dermal macrophages, but extracellular amastigotes are often seen (A-B). Magnification = 900x.



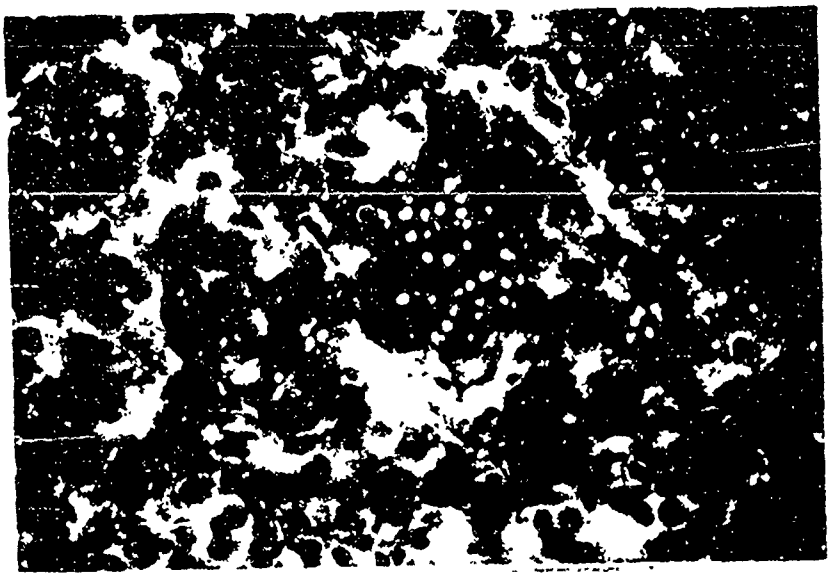
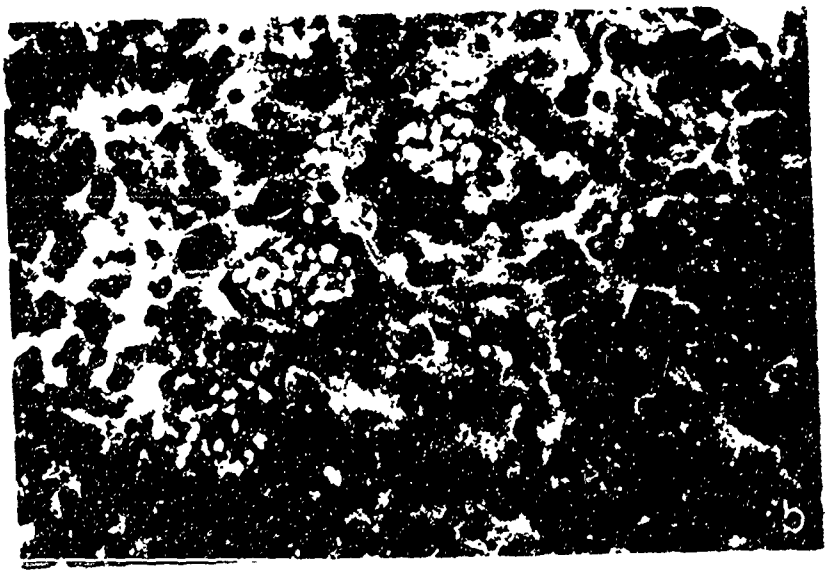


Fig. 2 Immunofluorescent localization of amastigotes in the popliteal lymph node of a Balb/c mouse 1 month after inoculation with L. m. amazonensis promastigotes. Frozen sections stained with monoclonal antibody 84C-4F4 as described in materials and methods. Numerous amastigotes within macrophages are easily visualized (A-B). Magnification = 900x.



5. USE OF FLOW CYTOMETRY FOR DETERMINING SURFACE ANTIGEN EXPRESSION:

Microfluoremetric flow cytometry has been developed as an alternative aproach to quantitate surface antigen expression of the various species and sub-species of New World Leishmania. In brief, this procedure entails :

a. Incubation of living promastigotes with the respective monoclonal antibody.

b. Addition of an FITC labeled goat anti-mouse immunoglobulin serum.

c. Enumeration of the number of parasites labeled, as well as the intensity of the label, in the fluorescent activated cell sorter.

Preliminary results are presented in the accompaning table.

TABLE I

QUANTITATION OF LEISHMANIA SURFACE MEMBRANE ANTIGENS ON THE BASIS OF THEIR REACTIVITY WITH MONOCLONAL ANTIBODIES IN FLOW CYTOMETRIC ANALYSES[a]

Antibody	*L.m.mexicana* WR 222	*L.m.amazonensis* WR 303	*L.m.amazonensis* GML 111	*L.b.guyanensis* WR 390	*L.species* WR 359	*L.b.panamensis* GML 1	*L.b.braziliensis* WR 508	*L.b.braziliensis* GML 18
P3 (Neg)	10.4	10.9	10.9	10.6	9.7	10.2	10.2	9.7
83H-2D6	13.3	13.7	14.7	13.4	12.8	12.9	13.8	12.0
84C-5B2	13.3	14.1	15.5	13.9	12.9	13.0	14.1	11.3
83T-6F11	10.1	10.4	10.6	9.7	9.8	10.0	10.1	10.4
84G-6B6	10.2	10.6	10.5	10.7	9.8	10.1	9.9	10.1
83T-9D3	95.6	106.4	91.1	54.2	21.8	10.1	10.9	9.7
83U-7D5	74.4	75.4	87.3	51.6	19.9	10.0	10.4	9.7
83U-2F11	66.0	85.5	84.8	46.7	14.9	10.1	9.9	9.7
83U-6F4	82.8	67.6	91.4	45.5	18.9	10.2	10.2	10.3
84C-8C7	74.4	96.4	82.5	53.3	18.4	10.3	10.9	9.5
83T-10E4	66.9	85.5	84.8	46.7	15.9	10.0	9.9	9.7
83U-5F2	36.8	38.3	32.4	20.3	10.8	10.0	9.8	9.7
83U-9B3	78.2	76.4	62.6	24.8	10.1	9.8	9.8	9.7

[a] Mean T2/T1 of 3 samples with 10,000 cells/sample analyzed.

Values > 16.0 considered significantly different from negative control by paired t-test.